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Identifying pathogenicity genes in the rubber tree anthracnose fungus *Colletotrichum gloeosporioides* through random insertional mutagenesis☆

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ABSTRACT

To gain more insight into the molecular mechanisms of *Colletotrichum gloeosporioides* pathogenesis, *Agrobacterium tumefaciens*-mediated transformation (ATMT) was used to identify mutants of *C. gloeosporioides* impaired in pathogenicity. An ATMT library of 4128 *C. gloeosporioides* transformants was generated. Transformants were screened for defects in pathogenicity with a detached copper brown leaf assay. 32 mutants showing reproducible pathogenicity defects were obtained. Southern blot analysis showed 60.4% of the transformants had single-site T-DNA integrations. 16 Genomic sequences flanking T-DNA were recovered from mutants by thermal asymmetric interlaced PCR, and were used to isolate the tagged genes from the genome sequence of wild-type *C. gloeosporioides* by Basic Local Alignment Search Tool searches against the local genome database of the wild-type *C. gloeosporioides*. One potential pathogenicity genes encoded calcium-translocating P-type ATPase. Six potential pathogenicity genes had no known homologs in filamentous fungi and were likely to be novel fungal virulence factors. Two putative genes encoded Glycosyltransferase family 28 domain-containing protein and Mov34/MPN/PAD-1 family protein, respectively. Five potential pathogenicity genes had putative function matched with putative protein of other *Colletotrichum* species. Two known *C. gloeosporioides* pathogenicity genes were also identified, the encoding *Glomerella cingulata* hard-surface induced protein and *C. gloeosporioides* regulatory subunit of protein kinase A gene involved in cAMP-dependent PKA signal transduction pathway.

1. Introduction

Rubber trees (*Hevea brasiliensis*) are perennial crops of Amazonian origin that have been spread over the whole tropical belt to guarantee worldwide production of natural rubber. This crop plant has found its place in many national economies of producing countries; it contributes a lot to the welfare of small farmers worldwide. Natural rubber (NR) produced from latex of the rubber tree *Hevea brasiliensis*, almost the sole source of natural rubber production, nowadays accounts for about 40% of the world's total rubber consumption; 60% is delivered by synthetic processes. The annual world demand for natural rubber is constantly growing because of its typical physicochemical properties, which are still not achieved by synthetic products, and wide range of industrial applications (Lieberei 2007).

Anthracnose disease caused by *Colletotrichum* (Gloeosporium) of the *Glomerella* group is very common and destructive for numerous crops and ornamental plants worldwide (Münch et al. 2008; Kim et al. 2002; Lubbe et al. 2004). Among them, *Colletotrichum* Leaf Disease (CLD) of rubber tree is considered as one of the major causes for the decline in yields of rubber in the Asian continent. CLD infects most organs of rubber tree, resulting in anthracnose on leaves, twigs, flowers and young fruits (Liu et al. 1987). The consequences of the disease are leaf necrosis and deformation, and even secondary fall of the youngest leaves affected at an early stage of their development. Consequently, new leaves produced throughout the rainy season are systematically destroyed by the disease. The trees become less vigorous as the flushes succeed each other, and the initial canopy density is therefore never re-established. The disease epidemic causes a substantial drop in foliage density and death of terminal branches, known as die-back (Jean et al. 2001). In China CLD is a severe problem and has become a limiting factor for rubber production. Huge yield losses due to CLD have been observed in Hainnan, Guangdong and Yunnan provinces when the

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weather was favour the disease (Cai et al. 2009). The pathogen, *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. have been proved to be the causative agent of CLD in all rubber-growing countries in the world since early 1900s (Thambugala and Deshappriya 2009; Samaradeewa et al. 1985; Petch 1906; Nicholas 2010; Jean et al. 2005).

Since 1965 extensive studies have been carried out on the taxonomy, population structure, epidemiological investigations, etc., but more attention should be paid to molecular pathogenicity mechanism of the disease (Cai and Huang 2001). Depending on genome size of the fungus and its manner of infecting plants, Idnurm and Howlett estimated that a phytopathogenic fungus has between 60 and 360 pathogenicity genes (Idnurm and Howlett 2001). However, only some of them have been identified. Our study was therefore, undertaken to discover the new pathogenicity genes by insertional mutageneses. Genes involved in pathogenicity have been identified in *C. gloeosporioides* based on their expression pattern (Kim et al. 2000b, 2002; Hwang et al. 1995; Stephenson et al. 2000; Liu and Kolattukudy 1998), by targeted deletion of genes encoding a known physiological function (Kim et al. 2000a; Goodwin et al. 2001), or by isolation of pathogenicity mutants (Barhoom et al. 2008). Of these approaches, isolating pathogenicity mutants clearly has the greatest potential for finding novel genes that encode determinants of pathogenicity. T-DNA tagging, a form of insertional mutagenesis that relies on AMT to mutate the recipient genome at random sites by integration of T-DNA carrying a selectable marker, has been developed as a powerful tool for both random and targeted gene disruption and is increasingly being seen as the system of choice for many fungi (Michielse et al. 2005), used successfully to find new genes (Li et al. 2005; Walton et al. 2005) and T-DNA tagging projects on fungi have recently been initiated in many laboratories around the world (Rogers et al. 2004). One of the principal advantages of ATMT over conventional transformation techniques is the versatility it provides in choosing which starting material to transform. *Agrobacterium tumefaciens* can transform protoplasts, hyphae, spores, and blocks of mushroom mycelial tissue (De Groot et al. 1998), produce larger numbers of stable transformants, and more single copy T-DNA insertions than conventional transformation methods (Weld et al. 2006). As the transformants are tagged with T-DNA, it is easy to identify the insertion site. Moreover, ATMT is simple, and suitable for both random insertional mutagenesis and targeted mutagenesis.

In this report, we describe the use of ATMT to generate a library of chlorimuron-ethyl-resistant transformants of *C. gloeosporioides* from which we have screened for mutants defect in pathogenicity. More than 4000 transformants have been screened to date. We established a reliable screen method for pathogenicity mutants and 32 mutants defect in pathogenicity obtained. Sixteen T-DNA integration sites of transformants impaired in pathogenicity were identified, and in most cases T-DNA integrated into a predicted ORF of putative genes based on gene structure prediction by FGENESH program (Softberry Inc., Mount Kisco, NY, USA, http://linux1.softberry.com/berry.phtml). Except two genes which had proved to be pathogenicity genes in *C. gloeosporioides*, the rest of putative genes were not previously implicated in the pathogenicity of any *Colletotrichum* species.

2. Materials and methods

2.1. Fungal and bacterial strains

The wild-type strain of *C. gloeosporioides* isolated from *Hevea brasiliensis* was used in this study as a recipient host for transformation, which was maintained on PDA at 25 °C. The *A. tumefaciens* AgL-1 was used as T-DNA donor for fungal transformation. The bacteria harboring pSULF-GFP originally developed by Sesma and associates (Fig. 1A) was kindly provided by C.Z. Hei (University of Hainan), which carries both the *ILV1* and green fluorescent protein (s*GFP*) genes. *Agrobacteria* were grown at 28 °C on Luria Bertani (LB) agar supplemented with 25 μg of rifampicin and 50 μg of kanamycin per milliliter.

2.2. Agrobacterium-mediated transformation and mutagenesis

Preparation of *A. tumefaciens* cells and fungal transformation and mutagenesis were conducted according to the protocols described by Mullins and associates with modifications (Mullins et al. 2001). Briefly, *C. gloeosporioides* conidial suspension (10^6 spores/ml) were mixed with an equal volume of *A. tumefaciens* cells diluted to an optical density at 660 nm of 0.4–0.5 in inducion medium (IM) broth supplemented with 200 μM acetosyringone at 28 °C for 6 h. This mix (200 μl per plate) was plated onto minimal medium (MM) containing 5 mM glucose, 0.5% glycerol, and 200 μM acetosyringone, with a layer of 0.45-μm pore nitrocellulose membrane in 9-cm petri dishes; and co-cultured at 28 °C for 2 days. The nitrocellulose membrane was transferred onto the DCM medium amended with chlorimuron-ethyl (50 μg/ml), cefotaxim (100 μg/ml), and tetracycline (50 μg/ml) and monitored daily for appearance of fungal colonies, transformant colonies were transferred to DCM plates containing 100 μg of chlorimuron-ethyl per milliliter.

To test for the mitotic stability of the integrated chlorimuron-ethyl resistance cassette, 30 randomly chosen transformants were cultivated on PDA without chlorimuron-ethyl, with weekly transfer to new plates. After four passages in the absence of chlorimuron-ethyl, transformants were grown on DCM plates containing chlorimuron-ethyl (100 mg/ml).

2.3. Establish a bioassay for large-scale screening of the pathogenicity mutants

Inoculation was carried out on detached rubber tree leaves (CATAS 7-33-97, one of the main clones in China) at two different developmental stages: copper brown leaves (young) and light green leaves (older). A comparison was made between two inoculation techniques: (i) leaves were wounded prior to inoculation, (ii) leaves remained intact, at different incubation temperatures (16, 20, 24 and 28 °C), assessed the effects of incubation temperature, leaf stage, inoculation technique, the conditions best suited for a rapid and reliable bioassay for screening of *Colletotrichum* mutants impaired in pathogenicity were determined, then performed pathogenicity assays as follow.

Pathogenicity assay. In a primary screen, infection assays, agar plug assays on intact, detached copper brown rubber tree (CATAS 7-33-97) leaves (approximately 5–7 cm in length) were used to screen for transformants that reduced in pathogenicity after *Agrobacterium*-mediated transformation. A 4-mm diameter agar plug was cut from mycelial mats cultured on PDA and placed onto rubber tree leaves with the fungal mycelium directly in contacted with the leaf surface. Mutants were further confirmed at least three times by inoculation using conidial suspensions. Inoculation assays using conidial suspensions were conducted as previously described by Cai and associates (Cai et al. 2012), with modifications. Briefly, fungal conidia were harvested by flooding 4-day-old cultures with sterile water from single conidial cultures, centrifuging, and adjusting the concentration to 1×10^6 conidia/ml, then 20 μl conidial suspensions were dropped onto intact, detached copper brown rubber tree leaves. Inoculated leaves placed on moist tissue paper were maintained in a moist chamber and incubated 28 °C, monitored daily for lesion development.

Defects in pathogenicity of some selected mutants which were conformed with intact, detached copper brown rubber tree leaves,